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14. ABSTRACT
The goal of this program is to investigate the influence of controlled mechanical stimulation on the behavior of progenitor cells in an effort to develop strategies to significantly enhance the rate and quality of fracture repair in long bones. In support of these goals, we will test the global hypothesis that the migration, proliferation and differentiation of systemically or locally delivered Mesenchymal Stem Cells is temporarily dependent on local mechanical conditions within the regenerate tissues.

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Introduction

The goal of this program is to investigate the influence of controlled mechanical stimulation on the behavior of progenitor cells in an effort to develop strategies to significantly enhance the rate and quality of fracture repair in long bones. In support of these goals, we will test the global hypothesis that the migration, proliferation and differentiation of systemically or locally delivered MSCs is temporarily dependent on local mechanical conditions within the regenerate tissues.

Body

The progress of this research program is described below, as a function of the statements of work that were approved by the USAMRMC. The statement of work was proposed as follows:

1. Acquisition of transgenic GFP rats and establishment of a small colony for cell donation. This will be accomplished in the first eight months of the study.

2. Extraction, isolation and expansion of MSC from transgenic GFP rats to establish baseline of GFP signal in culture. This will occur during year 1.

3. Delivery of MSCs from GFP rats into wild type rats after treatment with F^{18}. This will be a dosing and cell viability study using microPET imaging and will be accomplished during year 1.

4. Fabrication of the required external fixation devices, associated pins and surgical guides will be performed during years 1 through 3.

5. Implementation of the first primary experiment: 108 rats with bilateral femoral 2mm defects and fixation will be entered into the study to evaluate the effect of load and systemic cell delivery on cell migration, using microPET scanning. Animals will be entered in year 1 through year 2.

6. The evaluation of the effect of delivery and mechanical stimulation on bone regeneration using histologic, micro-imaging and biomechanical assays will be performed in years 1 through 2.5.

7. 144 animals will be entered into the second primary experiment to evaluate the effect of local cell delivery and mechanical stimulation during years 2.5 through 3.5.

8. Complete analysis of the combined effects of local or systemic cell delivery with mechanical stimulation will be completed during years 3.5 through 4.

During the first year of the study, we have made substantial progress in several areas as designated in the proposal timetable, while some areas were delayed due to unexpected regulatory issues. As a result, we have altered our studies in an effort to maximize progress. Essentially, we utilized the delay in one of the tasks to begin other tasks ahead of schedule, as will be described.

A. The first three tasks expected for completion during year 1 involved the acquisition of the GFP transgenic rats from the supplier in Japan. However, due to a new law imposed by the Japanese government, substantial new procedures were put in place to tightly regulate the export of any transgenic animal models from Japan. After learning of these new requirements we worked hard to meet the new rules and even utilized colleagues in Japan to help us interpret the laws and make sure we had no language barriers delaying our actions. As a result, we were finally able to acquire the animals, but at a time that was 9 months later than planned. We now have the animals, but the progress on the first three tasks is substantially altered as summarized below after each task description.

1. **Acquisition of transgenic GFP rats and establishment of a small colony for cell donation. This will be accomplished in the first eight months of the study.**

The animals have now been acquired and are residing in our vivarium at the University of Michigan. We have begun to breed the animals and we now anticipate that the first colony will be available for extracting donor cells by early 2007.

2. **Extraction, isolation and expansion of MSC from transgenic GFP rats to establish baseline of GFP signal in culture. This will occur during year 1.**

As noted above, the colony will be available in early 2007. At that time we will extract and culture the MSCs and verify their harboring the GFP construct. In the meantime, we have acquired non-transgenic Sprague Dawley rats and have verified our MSC extraction protocols and are ready from a procedural perspective.

3. **Delivery of MSCs from GFP rats into wild type rats after treatment with F^{18}. This will be a dosing and cell viability study using microPET imaging and will be accomplished during year 1**.

Similar to above, we will perform these studies in early 2007. In the meantime, we have begun extracting MSCs from non-transgenic rats and are utilizing SPECT imaging (with Indium ^{111}) which enables a longer time for imaging (3 to 4 days) as compared to PET using F^{18}. These current studies which will be complete within the next 2 months, will verify the time when the homing of the

progenitor cells are maximal. This will provide substantial help as we begin the PET studies and also verify that PET is the appropriate method for tracking the cells. These studies are progressing well.

B. The next tasks were designed to initiate the first *in vivo* primary studies of the effect of mechanical stimulation on the fracture repair process. Although we have not been able to start the arms of these studies that utilize the GFP donor cells, we have made substantial progress on these tasks and are on schedule. The details are described below.

1. Fabrication of the required external fixation devices, associated pins and surgical guides will be performed during years 1 through 3.

We have completed the design and have been fabricating the specialized external fixator systems that enable mechanical loading. One variation we have made in the devices has been the use of a radio-opaque material to allow improved radiography and important enable the possibility of using *in vivo* Micro CT. These fixators are now made from PEEK, an approved polymer that is already in use in humans. The devices are working extremely well. Photographs of the fixator as well as its implementation on animal are illustrated below.

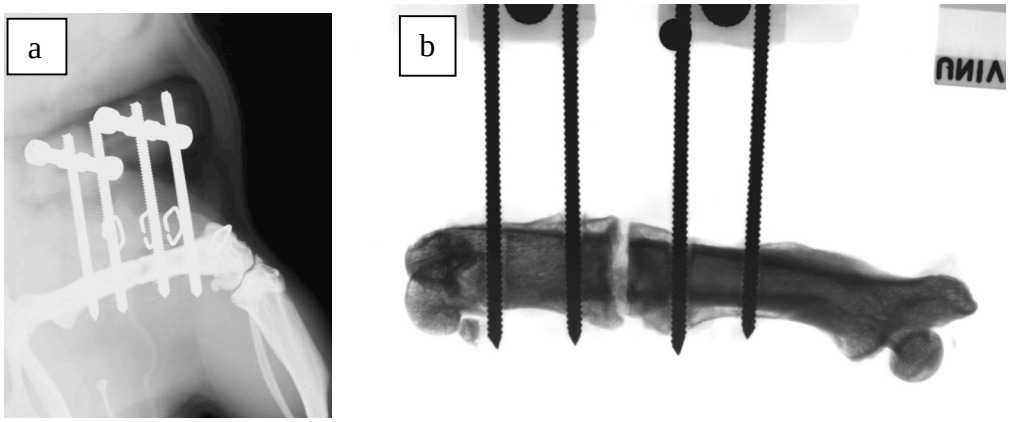

Figure 1: (a) The PEEK external fixator is illustrated as placed in one of the experimental animals. Since the fixator body is radiolucent, only the internal screws are visible in the radiograph. (b) As illustrated in the specimen from an animal that was evaluated for 3 weeks, the stabilized 2mm. defect does enable healing of the osteotomy.

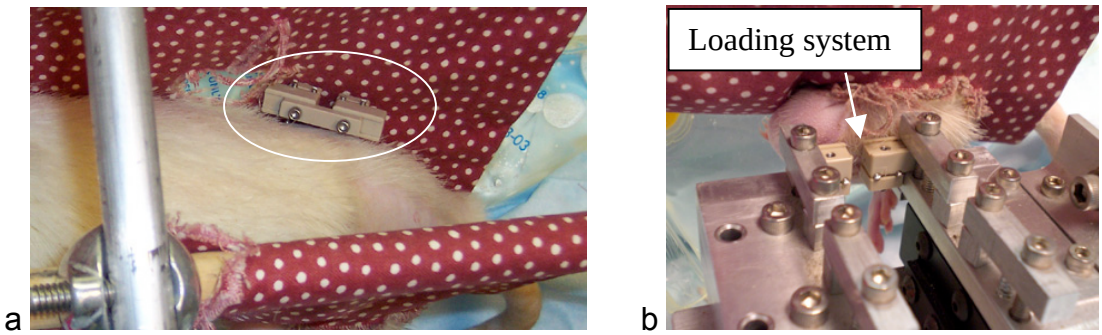

Figure 2: (a) This figure shows the animals constrained within the loading system. The PEEK external fixator is visible in the top portion of the photograph. It should be emphasized that the animals have bilateral implants. In this case, the left femur/implant is engaged in the loading system, while the right femur/implant remains unloaded. (b) The mechanism for locking onto the fixator and then applying load across the healing fracture is readily observed in the close-up.

2. Implementation of the first primary experiment: 108 rats with bilateral femoral 2mm defects and fixation will be entered into the study to evaluate the effect of load and systemic cell delivery on cell migration, using microPET scanning. Animals will be entered in year 1 through year 2.

As noted above, this portion of the timetable has been slowed by the delay in delivery of the GFP donor animals from Japan. To date we have entered 4 series of animals into the studies. The groups are associated with the verification of the loading parameters and verification of the outcome measure techniques. The major portion of the experiment is scheduled to begin within the next 2 months.

The results of the early animals have been evaluated. The loading system does alter the response of the fracture repair and these early results will be presented at the 2007 Orthopaedic Research Society meeting in San Diego.

3. The evaluation of the effect of delivery and mechanical stimulation on bone regeneration using histologic, micro-imaging and biomechanical assays will be performed in years 1 through 2.5.

As noted above, these studies will be commenced in “full force” during the next couple months.

Additional Studies

As noted above, the delay in receipt of the GFP transgenic rats from Japan caused us to alter the timetable and early focus of the program. While we are on schedule with respect to design, fabrication and evaluation of all the implants and proposed assays, we have not entered the large number of animals to date. They will commence within the next 2 months. Given these circumstances, we initiated studies to develop methods to identify the specific cells within the repair tissues that are responsive to mechanical stimulation. If successful, these studies will provide evidence for the cell populations that would be capable for responding to therapeutic intervention using mechanical stimulation. The specific studies and findings are summarized below.

Development of laser capture techniques to select and evaluate specific cell groups within the repair tissues.

During the past year as part of our exploration of robust methods for assaying the response of repair cells to mechanical stimulation, we developed protocols using laser capture technology. The advantages of laser capture include:

a. The ability to visualize and subsequently select specific cell or tissue regions for evaluation.
b. Prior to laser capture, the tissue sections can be stained to provide additional specificity above and beyond visual recognition (based on morphology) to identify and select specific tissue regions of cells.
c. The technology of laser capture has matured substantially, particularly with the introduction of new microscopic systems, over the last several years enabling precise selection of cells for analysis.

We have used laser capture to select cells for evaluation from regenerate tissue extracted from our rat model of fracture healing and mechanical stimulation. The specific refined protocol that we have developed is involves the following:

Fracture gap material was excised and embedded in OCT embedding medium (Tissue-Tek Inc.). Specimens were frozen by placing them directly onto an aluminum block that had been super-cooled in liquid nitrogen and were subsequently stored at -70° C until they were sectioned. Cryosectioning was performed in a Hacker-Bright OTF cryostat at -20° C. Sections were cut using a tungsten carbide blade and mounted onto a PEN Membrane Glass Slide (Arcturus Inc.) and stored at -70° C. For the preliminary studies, slides were fixed in 70% ethanol and immediately stained using a modified hematoxylin and eosin protocol. Following dehydration in 100% ethanol and xylenes, slides were air-dried and taken directly to be microdissected. Microdissection was performed using an Arcturus Veritas system. In brief, a CapSure Macro LCM cap coated with a thin “transfer film” is placed above a given region of interest. A UV cutting laser traces around the region of interest and an infrared laser is pulsed through the cap, melting the transfer film onto the cells of interest, thereby bonding the cells to the cap. The cap can then be directly visualized to confirm the completeness of the capture. An example of a tissue section demonstrating the region captured by laser dissection is illustrated in Figure 3.

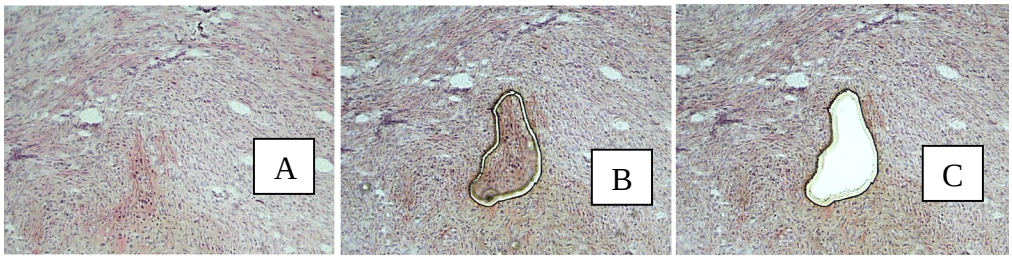

Figure 3: An example of laser capture microdissection is illustrated. A portion of the fracture repair tissue at 7 days was subjected to the laser capture technique. (A) A

region of granulation tissue dominantly composed of fibroblastic-like cells was selected. (B) Using the laser, the "cut" region is demonstrated, and (C) then extracted as described above.

Following capture, caps were inserted into a 0.5 mL RNase free tube containing 100 ml of Buffer RLT (Qiagen). The tubes were inverted, ensuring that the cap was covered in buffer, and incubated at 42° C for 30 minutes. The caps were then gently vortexed and centrifuged at 2000g for 1 min. An additional 250 ml of Buffer RLT + 1% b-mercaptoethanol was added to the samples, as well as 20 ng carrier RNA (Qiagen). Samples were then vortexed for 30 seconds and stored at -70° C until further processing.

RNA was extracted using an RNeasy Micro Kit (Qiagen) based on the manufacturer's protocol. Following RNA elution, 10 ml of RNA was reverse transcribed using standard methods. PCR was performed and amplification of gene products was visualized after staining in ethidium bromide. b-actin was used as an internal control for normalization

Pilot studies using laser capture techniques

10 Sprague Dawley rats were entered into the study and had bilateral insertion of the external fixators as described earlier. Briefly, the procedures involved the placement of the external fixators on both femurs and the creation of 2mm segmental osteotomies. After normal cage housing and activity for 7, 10 or 14 days, all animals were subjected to axial loading on one randomly selected limb. The loading regiment included a sinusoidal waveform, at 0.5Hz frequency with a maximum controlled displacement that corresponded to gross maximum average strain of between 5 and 8% (dependent on specific group) in the regenerate tissue. Each loaded limb/regenerate was loaded for a total of 510 cycles. After completion of the single loading regiment, each of the regenerate tissue regions was dissected free en-bloc (both loaded and contralateral unloaded) and prepared for laser capture microdissection. The timing for the extractions was controlled such that the tissue was embedded in the OCT and super-cooled at 15 minutes after the completion of the loading sequence and therefore, approximately 35 minutes from the initiation of the first loading cycle.

As illustrated in Figure 4, we chose two specific populations of cells from the regenerate tissue to examine; fibroblastic-like cells and chondrocytes. Laser microdissection was used to isolate the two cell groups and the cells were processed using RT-PCR. Our focus and the primary outcome marker for these studies and the proposed program is **c-fos.** C-fos was chosen based on extensive literature demonstrating its up-regulation in response to load (as well as many other perturbations) and our own substantial experience demonstrating the upregulation of c-fos by mechanical load in our earlier *in vivo* studies.

The results of the study demonstrated several important issues. 1) We were easily able to select the different cell types to test for their mechanoresponsiveness, 2) The laser capture technology and using PCR enables us to characterize c-fos (as well as other) transcription factor activation in response to load. Most interestingly, the preliminary data suggests that the chondrocytes are responding to load while the fibroblastic cells demonstrate no differences in c-fos activity compared to the unloaded cells. This is illustrated in Figure 5. As noted, using b-actin as a background control, the chondrocytes from the loaded fracture site illustrated up-regulation of c-fos while the chondrocytes from the contralateral unloaded side as well as the loaded or unloaded fibroblasts showed essentially no response with respect to c-fos.

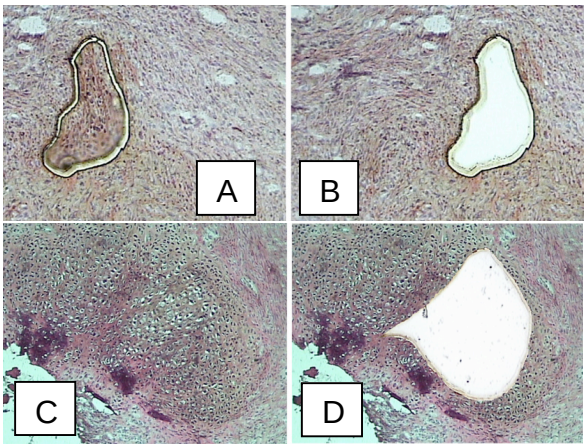

Figure 4: Fibroblastic cells were captured from one region of the repair tissue (A/B), while chondrocytes were selected and evaluated from a different location (C/D).

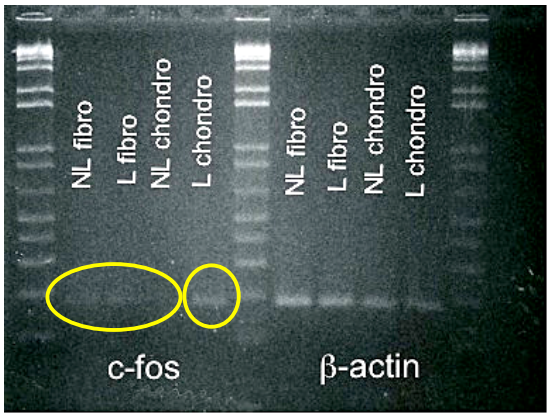

Figure 5: PCR results demonstrated that the chondrocytes from the loaded fracture site (L chondro) responded by upregulating c-fos. Chondrocytes from the unloaded side and fibroblasts from loaded or unloaded sites had little or no response.

Key Research Accomplishments

- Novel and effective external fixators have been fabricated and utilized to stabilize 2mm. defects in rat femurs. The devices include removable locking plates to enable mechanical stimulation of repair tissue.
- PEEK fixators allow for unobstructed imaging of defects.
- GFP transgenic rats have been acquired from Japan for donor progenitor cells
- Initial studies indicate effective response to mechanical stimulation.
- Development of laser microdissection techniques to isolate specific cell populations for examination of their responsiveness to mechanical simulation. Measurement of c-fos transcription regulation utilized as marker for mechano-response

Reportable Outcomes

An abstract has been accepted for presentation from these first year studies.

Pagedas CM, Miller JD, Weaver AS, Joiner DM, Kreider J, Goldstein SA: Assessment of cell-specific mechanoresponsiveness in fracture repair tissue in vivo. ORS 53rd Annual Meeting, February 11-14, 2007, San Diego, CA.

Conclusion

As summarized in the body of the report, the results from the first year studies have verified our experimental model, established a new method of identifying mechanoresponsive cells and begun to establish our labeled (GFP) progenitor cell donor population.

References

None

Appendices

None